IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In Re Application Of: Frame, Anne D.	)	Group Art Unit:	1655
	)		
Serial No.: 10/500,098	)	Examiner:	Patricia A. Leith
	)		
Filed: 06/24/04	)	Attorney Docket No.:	083622/00003
	)		
For: Anti-bacterial Plant Compositions	)		

DECLARATION OF SHELDON E. BROEDEL UNDER 37 C.F.R. §1.132

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

I, Sheldon E. Broedel, declare and state:

1. I am the Chief Science Officer at Athena Environmental Sciences, Inc.; a Member of the Advisory Board to the College of Natural and Mathematical Sciences (previously Founding Chairman of the Advisory Board) at the University of Maryland, Baltimore County ("UMBC") campus; a Scientific Advisor to Meyer Pharmaceuticals, Inc.; a Scientific Advisor to Stevenson University (Baltimore); Adjunct Professor in the Department of Microbiology and Immunology at the Georgetown University School of Medicine; and Adjunct Instructor, Graduate School for the Department of Biological Sciences, UMBC.

2. I have worked in the field of Biological Sciences in research in academic and industrial settings for 30 years. In the year 1990 I was awarded a doctoral degree from Department of Biological Sciences, UMBC. In the year 2000 I was awarded a Certificate from the Biochemical Regulatory Engineering Program at UMBC. During these 30 years I have often had occasion to conduct and supervise collaboratory research including interdisciplinary team

members, and including organic chemists and biologists involved in anti-infective drug development. My *Curriculum Vitae* is attached as Exhibit 1.

3. I have read the originally filed U.S. Application Ser. No. 10/320,492 ("the '492 Application") which issued as Patent No. 7,641,919. I understand that the '492 Application shares an identical specification with co-pending U.S. Application Ser. No. 10/500,098, 12/653,146, i.e., the continuation of the '492 Application, and with PCT/US01/50502 (published as WO 03/059371), and entries into national/regional phases off this PCT application as well as continuation and divisional applications of any of the above applications. I have also read selected recent rounds of correspondence between the USPTO and the applicant's representative in the pending U.S. Applications. I understand that similar issues may arise in all these patent applications in the U.S. and elsewhere, which share the same specifications and my declaration applies to all these matters.

4. I note that the specification indicates that a plant material is extracted by an organic solvent, preferably methylene chloride, and the material is further purified by HPLC. An HPLC fraction is selected as having anti-mycobacterial effect. The Applicant identified cobaltocene octamethyl as an ingredient of that fraction having the anti-mycobacterial effect. I undertook the synthesis of cobaltocene octamethyl and its testing, with appropriate controls, to determine if synthetically prepared cobaltocene octamethyl (i.e. free of other plant derived ingredients) has anti-mycobacterial activity. I have collaborated with Dr. Aristotle Kalivetrenos, an organic chemist in this project. Dr. Kalivetrenos' *Curriculum Vitae* is attached as Exhibit 2. I am the principal scientist and the person managing this project.

5. The results are presented in Exhibit 3. I conclude that cobaltocene octamethyl was synthesized by a known method and all tests indicate a successful synthesis. Two

preparations of cobaltocene octamethyl, each in a different solvent, were tested for activity against *M. smegmatis* by microbial susceptibility assays. The solvents and the starting materials were used as controls. Unequivocally, both preparations of the cobaltocene octamethyl material showed anti-mycobacterial activity. Neither of the solvents nor the starting materials tetramethylpentadiene and NH_4PF_6 had any observable activity. $CoCl_2$ was used as a positive control and showed activity against *M. smegmatis*

6. I declare further that all statements made herein are of my own knowledge and are considered by me to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Respectfully submitted,

Date: 2/18/10

Sheldon E. Broedel

I. Chemical Synthesis

Cobaltocene octamethyl was prepared in accordance with the literature (Kolle, U. and Khouzami, F. *Chem. Ber.* 1981, *114*, 2929-2937).

Synthesis

Materials

Starting materials were obtained either from the Aldrich Chemical Co. or from other commercial suppliers and were used as obtained, unless otherwise noted. NMR spectra were recorded at 400 MHz for ^{1}H, and at 100 MHz for ^{13}C using tetramethylsilane (TMS) as the internal reference. IR spectra were recorded on a Jasco FT/IR-4200 spectrometer equipped with an ATR cell. RP-HPLC was performed using a Dionex D500 chromatography system equipped with a GP40 gradient pump, an AD20 UV/Vis detector, a Millipore column heater, Vydac Denali column (C_{18} silica, 5 micron particle size, 120 A pore size, 4.6 x 250 mm) and controlled by Dionex PeakNet software v4.3.

RP-HPLC Conditions:

Sample:	dissolved in Solvent and filtered @ 0.45 microns
Solvent:	95/5/0.1 H_2O/MeCN/TFA
Flow rate:	1 ml/min
Temperature:	30°C
UV:	300 nm
Injection:	0.100 ml

Method

Preparation of cobaltocene octamethyl hexafluorophosphate (1).

To a dry 100 ml round bottom flask equipped with a septum closure and kept under 1 atmosphere of N_2 gas was added 0.50 g (4.09 mmol) of tetramethylcylcopentadiene in 50 ml of anhydrous tetrahydrofuran (THF). The solution was cooled to -70 C followed by the dropwise addition of 2.81 ml of 1.6 M solution of nBuLi in hexanes (4.50 mmol, 1.1 equiv). The reaction was kept at -70 C for 30 min and then allowed to warm to ambient temperature and stirred for 4 h. At this time, the reaction mixture was cooled to -70 C followed by the addition of 0.294 g of $CoCl_2$ (2.21 mmol, 0.54 equiv) as a solid in one portion. After 30 min, the cold bath was removed and the reaction mixture was allowed to stir at ambient temperature for 15 h. To the resulting dark solution was added a solution of 0.373 g Fe(III)Cl_3 in 0.83 ml of conc. aqueous HCl. The mixture immediately turned green. The solution was transferred to a 250 ml round bottom flask and concentrated in vacuo. To the dark green residue was added 10 ml of water. The aqueous solution was extracted with 2-20 ml portions of diethyl ether. To the resulting yellow aqueous extract was added 0.371 g of NH_4PF_6 (2.21 mmol, 0.5 equiv.). The pH of the aqueous solution was carefully adjusted to pH 6 by the addition of 1 N aqueous NaOH solution. resulting in the formation of a brown precipitate. The precipitate was isolated by centrifugation,

and washed twice with water and once with acetone. The resulting brown solid was allowed to dry at ambient temperature. Two essentially similar, synthesis preparations were undertaken.

Analysis

FT-IR (ATR cell): 3550, 3173, 3060, 1632, 1427, 1020 cm^{-1}.

RP-HPLC: peak with retention time at 3.82 minutes.

^{1}H NMR (DMSO-d6): δ 7.3 (bs), 1.24 (s).

Results and Conclusions

Cobaltocene octamethyl salt was prepared according to the literature (Kolle, U.; Khouzami, F. *Chem. Ber.* 1981, *114*, 2929-2937). The resulting brown powder product was characterized by mass spectra, FT-IR and ^{1}H NMR. Each method of characterization was consistent with the conclusion that the desired cobaltocene octamethyl compound was produced. The RP-HPLC analysis indicates a homogeneous product – a simple peak was produced.

II. Microbial Testing

Materials

Mycobacterium smegmatis strain VT30

Solid Middlebrook Medium – BD Bioscience 295964 lot 9223052

Middlebrook Broth – BD Bioscience 271310 lot 9076359 supplemental with ADC 211887 lot 915424

Synthetic Cobaltocene – two preparations, see above.

Dimethyl Formamide (DMF) – Sigma D-8654 lot 89H3645

Dimethyl Sulfoxide (DMSO) – Sigma D-8779 lot 89H3636

$CoCl_2$, 100 mg/ml dissolved in Middlebrook broth

Tetramethylcyclopentadiene, 100 mg/ml dissolved in DMSO (TMPD)

Methods

Two preparations of cobaltocene were tested for anti-mycobacterial activity. For the anti-microbial testing, the first preparation was suspended in DMSO. For the second preparation, 10.1 mg aliquot was suspended in 1.01 ml of DMF. The absorbance at 605 nm of the dissolved material was determined. The absorbance was 2.7 AU.

A disk diffusion assay was used to determine if there is anti-mycobacterial activity. Each of the material solubilized in DMSO (preparation 1) and DMF (preparation 2) were tested. All tests were done in duplicates. A single colony of strain VT307 was used to inoculate 2 ml of Middlebrook broth and the culture incubated at 37°C for 24 hours. A 0.1 ml aliquot of the culture was evenly spread onto solid Middlebrook medium and allowed to dry for 10 min.

Sterile 6 mm filter disks were placed on the agar surface. To the respective disks, 10 µl of each sample (≤ 1 mg) was applied. The plate was incubated at 37°C for 48 hours and the size of the zone of inhibition determined.

Results and Conclusions:

Table 1 list the size and type of zone of inhibition obtained for each material. Neither of the solvents used to dissolve the cobaltocene preparations exhibited anti-mycobacterial activity. Likewise the TMPD and the NH_4PF_6 compound used to prepare the cobaltocene did not have activity. A 1 mg aliquot of the $CoCl_2$ was used as a positive control and showed inhibitory activity. Both preparations of the cobaltocene octamethyl had anti-mycobacterial activity. The clear zones suggest that the compounds are probably microbicidal.

Figure 1. Zones of inhibition after 48 hours incubation.

Table 1. Zones of inhibition.

Sample	Zone Size (mm)	Zone Type
DMF	0	-
DMSO	0	-
1 mg TMPD	0	-
1 mg NH_4PF_6	0	-
1 mg $CoCl_2$	15	Clear
Cobaltocene, Preparation 1	12	Clear
Cobaltocene, Preparation 2	13	Clear